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Citation

Huebsch, N., C. J. Kearney, X. Zhao, J. Kim, C. A. Cezar, Z. Suo, and D. J. Mooney. 2014. "Ultrasound-Triggered Disruption and Self-Healing of Reversibly Cross-Linked Hydrogels for Drug Delivery and Enhanced Chemotherapy." *Proceedings of the National Academy of Sciences* 111 (27) (June 24): 9762–9767. doi:10.1073/pnas.1405469111.

Published Version

doi:10.1038/ncomms4121

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Ultrasound-triggered disruption and self-healing of reversibly cross-linked hydrogels for drug delivery and enhanced chemotherapy

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Edited by Robert Langer, Massachusetts Institute of Technology, Cambridge, MA, and approved June 3, 2014 (received for review March 24, 2014)

Biological systems are exquisitely sensitive to the location and timing of physiologic cues and drugs. This spatiotemporal sensitivity presents opportunities for developing new therapeutic approaches. Polymer-based delivery systems are used extensively for attaining localized, sustained release of bioactive molecules. However, these devices typically are designed to achieve a constant rate of release. We hypothesized that it would be possible to create digital drug release, which could be accelerated and then switched back off, on demand, by applying ultrasound to disrupt ionically cross-linked hydrogels. We demonstrated that ultrasound does not permanently damage these materials but enables nearly digital release of small molecules, proteins, and condensed oligonucleotides. Parallel in vitro studies demonstrated that the concept of applying temporally short, high-dose “bursts” of drug exposure could be applied to enhance the toxicity of mitoxantrone toward breast cancer cells. We thus used the hydrogel system in vivo to treat xenograft tumors with mitoxantrone, and found that daily ultrasound-stimulated drug release substantially reduced tumor growth compared with sustained drug release alone. This approach of digital drug release likely will be applicable to a broad variety of polymers and bioactive molecules, and is a potentially useful tool for studying how the timing of factor delivery controls cell fate in vivo.

self-healing materials | alginate | on-demand delivery | sonophoresis

Polymer-based systems for drug delivery typically are engineered to yield a constant rate of release of bioactive compounds (1). Emergent clinical situations (e.g., pain attacks or infections) may create a need for significantly different drug doses over short periods, and in some cases, circadian drug timing increases effectiveness (2, 3). Likewise, controlled-release systems may be applied to dissect roles for specific physiologic cues or drugs during tissue development or regeneration, as these signals often are highly transient and may play opposite roles, depending on the timing of activation (4–7). Typical drug delivery systems that rely on polymer degradation and passive diffusion to control release allow precise spatial localization of cues, but not the ability to temporally control release rates at will. In contrast, on-demand release systems, such as “smart” polymers or materials activated by external stimuli [e.g., magnetic fields, radio waves, or light (8–13)] potentially offer this temporal flexibility and are beginning to be applied in the clinical setting (14). Ultrasound commonly is used in medicine and can deliver focused energy of varying magnitude and frequency with high spatiotemporal resolution (15). In particular, ultrasound has been used to trigger release from polymers by causing their degradation (16, 17). This degradation permanently changes the delivery vehicle, thus altering the baseline rate of molecular release as well as the release achieved with subsequent exposure to ultrasound. Interestingly, ultrasound also is used in a process known as sonophoresis, in which sound waves are applied to

disrupt skin transiently for needle-free transdermal drug delivery (15, 18). A crucial difference between sonophoresis and ultrasound-stimulated polymer degradation is that the damage induced by cavitation during sonophoresis is only temporary, because skin has a self-healing capability that switches delivery off following removal of the ultrasound stimulus. Because self-healing causes skin to revert to its basal state following removal of the ultrasound stimulus, repeated sonophoresis yields similar doses during each pulse (15, 18). A hydrophobic coating on a drug-containing polymer disk was shown to be disrupted by ultrasound and subsequently to reform on the polymer returning release to baseline; however, a self-healing coating is susceptible to replacement by nonspecific adhesion of proteins and cells in vivo, which would result in unpredictable release kinetics (19). Thus, we sought to develop an injectable monolithic system that can hold more drug per unit volume, responds to ultrasound, and subsequently self-heals. We first demonstrated that monolithic alginate hydrogels exhibited such behavior, and that this enabled on-demand delivery of drugs, cytokines, and condensed plasmid DNA (pDNA). In vitro experiments demonstrated that human breast cancer cells were more sensitive to high-dose “pulses” of the chemotherapeutic drug mitoxantrone than they were to the

Significance

Drug-releasing polymers give clinicians the ability to deliver chemotherapy directly to tumors, sparing the rest of the body from toxic side effects. Most devices deliver a constant, unchangeable drug dose over time. However, we found that cancer cells are more sensitive to short-term, high-dose “bursts” of the chemotherapeutic mitoxantrone than to constant doses over longer periods, suggesting a benefit for implantable devices that allow external control over dose and timing. Biocompatible, injectable alginate hydrogels displayed the ability to self-heal damage induced by ultrasound pulses, enabling on-demand delivery of mitoxantrone, in vitro and in vivo, and mitoxantrone-loaded gels implanted near tumors were more effective at eliminating tumor growth when a daily pulse of ultrasound was applied.

Author contributions: N.H., C.J.K., X.Z., Z.S., and D.J.M. designed research; N.H., C.J.K., X.Z., J.K., and C.A.C. performed research; N.H., C.J.K., and X.Z. contributed new reagents/analytic tools; N.H., C.J.K., and X.Z. analyzed data; Z.S. and D.J.M. supervised research; and N.H., C.J.K., and D.J.M. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1405469111/-DCSupplemental.

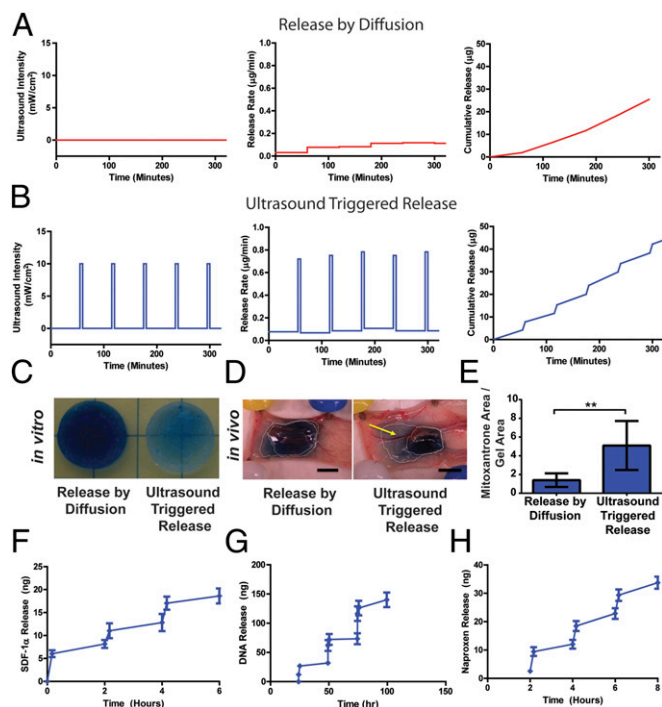


Fig. 2. Ultrasound-mediated on-demand release from ionically cross-linked hydrogels. (A and B) Quantitative analysis of mitoxantrone release via diffusion (A) or ultrasound treatment (B; 5 min, applied once every hour). Data indicate ultrasound intensity (Left), mitoxantrone release rate (Center), and cumulative mitoxantrone release (Right). (C) Representative images of residual mitoxantrone (blue) contained in alginate hydrogels after they were treated with ultrasound once per hour over 10 h (Right) or not treated with ultrasound (Left). (D) Representative images of residual mitoxantrone contained in alginate hydrogels, and mitoxantrone released from gels and subsequently deposited into host tissues, after hydrogels were transplanted into Nu/J mice and treated once with ultrasound for 2.5 min (Right) or left untreated (Left) over a 24-h period. (E) Quantitative analysis of the extent of in vivo mitoxantrone release, determined by the total surface area of s.c. tissue displaying visible staining with the drug (blue) normalized to the area of the transplanted gels. (F) Cumulative delivery of SDF-1 α from hydrogels that were treated with ultrasound for 10 min once every 2 h. (G) Cumulative delivery of pDNA condensed with poly(ethylene imine) from hydrogels that were treated with ultrasound for 10 min once every day. (H) Cumulative delivery of naproxen from TPP-chitosan hydrogels treated with ultrasound for 10 minutes once every 2 h. Error bars: SD, $n = 3-4$. * $P < 0.05$, t test.

Ultrasound treatment significantly enhanced naproxen release from these materials (Fig. 2H). Accordingly, these data suggest that a wide variety of bioactive molecules might be released in a pulsatile fashion, given the appropriately selected drug-gel pairing. With the appropriate choice of drugs and/or carrier molecules, one may be able to integrate ultrasound-mediated drug delivery into complex devices that release multiple compounds over different time frames (14).

As cancer cells specifically have been shown to exhibit dynamic responses to drug treatment (3, 4), we hypothesized that intermittent, higher pulses of drug dose might enhance the effects of chemotherapeutic drugs such as mitoxantrone. To test this hypothesis, we first analyzed the effects of controlling the timing of dose profiles on mitoxantrone-treated cancer cells in vitro. Two human breast cancer lines (MDA-MB-231 and MCF7) were treated with two different dose profiles of drug: (i) an intermediate, constant dose or (ii) a combination of short duration, high concentration of drug with sustained exposure to a lower baseline dose. Drug concentrations were altered via media exchange to prevent confounding of the study results by exposing cells to

ultrasound or to drugs released from gels via ultrasound, and with every media change, all samples were washed thoroughly with PBS and fresh media was added to avoid any confounding effects of residual drug (from previous dosings) or media exchange on cell viability. The integrated value of drug dose over time (e.g., total drug exposure) to which cells were exposed was maintained constant. Interestingly, the combination of temporally short exposure to a burst of drug with a low baseline dose appeared to be much more effective than sustained exposure to mitoxantrone in reducing cancer cell viability (Fig. 3A–C). Additional studies indicated that the total drug exposure also significantly contributes to the effects of mitoxantrone (Fig. S6). These results are consistent with previous work indicating that the dynamics of drug exposure have substantial effects on cell-level responses (4, 5), highlighting the importance of studying cell response to drug dynamics and the necessity for robust systems that facilitate these delivery modes in vitro and in vivo.

We then analyzed how the dynamics of mitoxantrone exposure affected viability in these cells. MDA-MB-231 cells were used in these studies, as they represent a more metastatic population than MCF7 breast cancer cells. The enhanced ability of a pulse of mitoxantrone to diminish cell viability was consistent across several dosing profiles (Fig. 3D). Further, increasing the time of burst exposure while diminishing the concentration during burst (to maintain the same level of integrated drug exposure) diminished the effect of mitoxantrone, whereas using an even shorter burst time with a higher dose enhanced its effects (Fig. 3D).

Having established the potential therapeutic utility of combining short bursts of concentrated drug release with a baseline, lower dose of drug in vitro and identifying a set of biomaterials that would allow such profiles to be delivered in vivo, we investigated the effects of ultrasound-mediated mitoxantrone delivery on treatment of xenograft human breast tumors (MDA-MB-231) in nude mice. First, short-term studies were performed to identify effects of ultrasound-mediated drug delivery on tumor growth and apoptosis. TUNEL staining of tumors after 8 d of treatment revealed that implanting mitoxantrone-laden alginate matrices led to significant apoptosis in the xenografts, regardless of whether a daily dose of ultrasound was applied to induce burst release (Fig. 4A and B). In contrast, systemic drug delivery of the equivalent amount of drug delivered via bolus tail vein injection did not lead to substantial apoptosis. This finding is consistent with literature reports of the poor ability of systemically delivered mitoxantrone to reach xenograft tumors [$<5\%$ of systemic dose typically found at xenograft hindlimb tumor site (25)]. Interestingly, daily local drug injections, or a bolus drug injection combined with daily ultrasound, yielded little to no improvement in xenograft apoptosis over tail vein injection. Although these results do not rule out the possibility that ultrasound might enhance tissue permeability, thereby increasing drug potency, they strongly suggest that a locally deployed drug depot is required to maintain a baseline concentration of bioactive drug. Consistent with this assumption, measurements of relative tumor size revealed that both a daily local injection of drug and the combination of basal delivery from a hydrogel combined with a daily ultrasound pulse led to a reduction in tumor size, but only the combined regimen yielded tumors that were statistically different in size than tumors from mice subjected to a systemic (tail vein) dose (Fig. 4C). Combined with the finding that drug-laden gels, with or without ultrasound pulsing, yielded significant levels of xenograft apoptosis, this suggests that the levels of mitoxantrone to which tumors are exposed are sufficient for eliminating tumors, but that a basal level of apoptosis is reached earlier in tumors in which drug-laden gels are pulsed with ultrasound, which translates into a substantial reduction in tumor mass. Thus, these data suggest that combining a local, basal release with intermittent

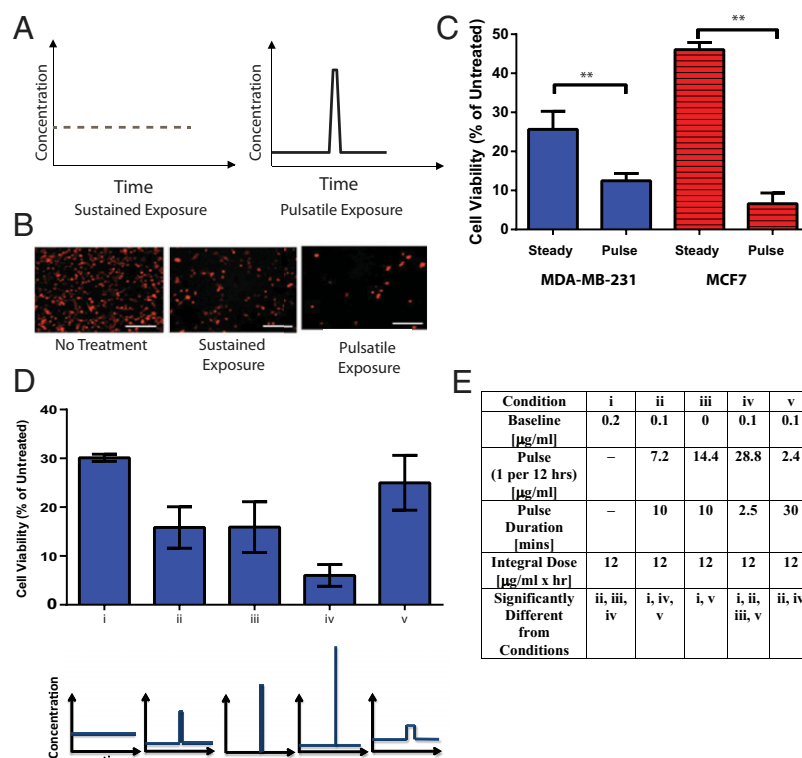


Fig. 3. Dynamics of mitoxantrone exposure affects drug effects on viability of human breast cancer cells in vitro. (A) Schematics depicting constant drug concentration, representative of the dose that would be expected with standard drug-delivering biomaterials (*Left*), or a lower baseline dose, interspersed with a high dose presented in a pulsatile fashion (*Right*), representative of the dose expected with on-demand release. Note that the integrated dose is the same. (B) Representative fluorescence micrographs of mCherry-labeled MDA-MB-231 breast cancer cells exposed to a sustained mitoxantrone dose (*Center*) or a lower baseline drug dose interspersed with a periodic high dose, applied for 10 min once every 12 h (*Left*). (C) Quantitative analysis of cell viability in MDA-MB-231 (blue) and MCF7 (red) breast cancer cell lines treated with sustained or pulsatile exposure to mitoxantrone. The cycle was performed once every 12 h and repeated five times, and the total drug exposure was constant at $12 \mu\text{g}\cdot\text{mL}^{-1}\cdot\text{h}^{-1}$ in both conditions. (D) Quantitative analysis of MDA-MB-231 viability after 60 h of various mitoxantrone dosing regimens. (E) Table detailing the dosing regimens used for D. Error bars: SD, $n = 4$ (** $P < 0.05$, two-tailed t -test), $n = 3-9$. Scale bars (B): 200 μm .

pulses of drug enhances treatment of tumors throughout the course of therapy.

Based on the results of our short-term studies, subsequent long-term studies on tumor growth and host survival were performed using locally deployed alginate gels. Tumors within mice receiving no therapy grew rapidly, and these animals succumbed to the effects of tumors within 100 d. Treatment with mitoxantrone-eluting gels alone decreased tumor growth rate and improved survival, as expected, with the time to 50% animal mortality increasing from 100 to 120 d (Fig. 4 *D* and *E*). Strikingly, the combination of low-level sustained release with a daily concentrated pulse of mitoxantrone delivered via ultrasound reduced the growth rate of xenograft tumors compared with treatment with mitoxantrone-laden gels alone (Fig. 4*D*). Importantly, this treatment improved survival of mice bearing these tumors after all treatments had stopped, with the time to 50% animal mortality nearly doubling from the value of 100 d for mice receiving no therapy to 180 d (Fig. 4*E*). Kaplan-Meir analysis revealed a statistically significant survival improvement in mice treated with either drug-laden gels or drug-laden gels exposed to a daily pulse of ultrasound, compared with mice receiving no therapy. Although survival did not improve significantly when daily ultrasound pulses were applied on top of the drug-laden gels, tumors were significantly smaller in this treatment group (compared with tumors in mice receiving only drug-laden gels) from days 10 through 70 of treatment (with the exception of days 24–31 and days 59–63). Notably, the drug payload within ultrasound-treated gels is depleted faster than the drug within gels loaded with drug

but not exposed to this stimulus (Fig. S7)—hence, mitoxantrone-laden gels not treated with ultrasound continued to release drug for a longer period; thus, the net amount of drug delivered to tumors over the course of this study was the same between these conditions. These results suggest that although localized mitoxantrone delivery is therapeutic, its effectiveness—and likely the effectiveness of other existing drugs—may be improved with optimal spatial as well as temporal delivery.

At the end of the 6-mo study period, surviving mice were euthanized and the masses of remaining tumors were analyzed. Tumors within surviving mice that had received a combination of ultrasound and mitoxantrone-laden hydrogels had not grown substantially since the start of treatment (Fig. 4F) and had a net mass of 63 ± 45 mg. In contrast, among tumors retrieved from surviving mice that had received mitoxantrone-laden hydrogels but not ultrasound, only one of the remaining tumors weighed less than 100 mg, whereas the other weighed nearly 1 g, or the approximate mass of tumors explanted from drug-treated mice that succumbed to tumors earlier in the study ($1,100 \pm 800$ mg).

Although the current studies demonstrate the potential of ultrasound-stimulated drug release to enhance chemotherapy, it is noted that further testing mimicking the exact application is required before its clinical adoption. The ultrasound intensities are within those used by other authors for ultrasound-stimulated drug release and were demonstrated to be safe in animal testing (17, 18). More sophisticated ultrasound devices that focus the signal on the gel would allow deeper implantation of the gel and mitigate any potential side effects. Such devices also would allow

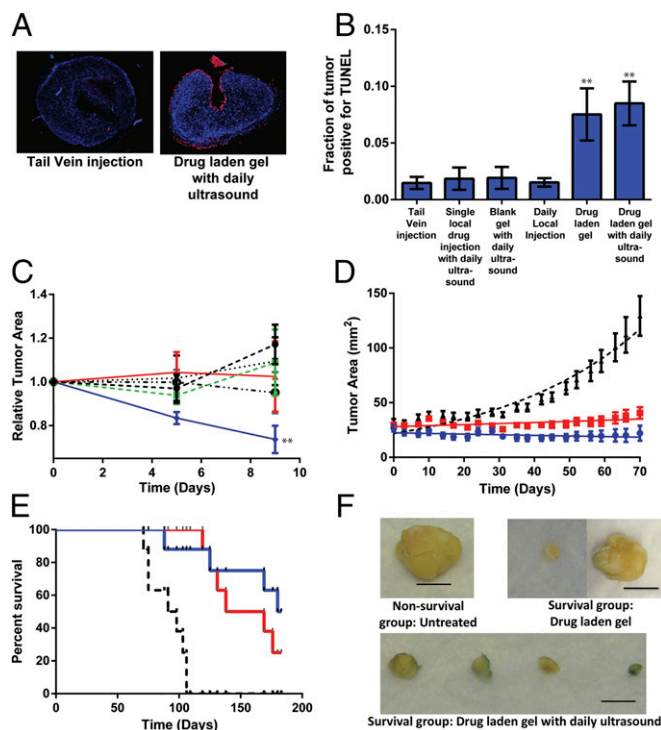


Fig. 4. Ultrasound-mediated local drug delivery diminishes xenograft tumor growth. (A–C) Short-term studies of xenograft tumor growth and apoptosis when treated with ultrasound-triggered mitoxantrone. (A and B) Representative images (A) and quantitative analysis (B) of TUNEL-stained tumor sections from mice bearing tumors that were treated via systemic tail vein mitoxantrone bolus or with various local delivery regimens. (C) Normalized area of tumors treated via systemic mitoxantrone dose (single tail vein injection of the maximum tolerated dose, 82.5 μ g; black \bullet); local injection of the maximum tolerated dose of mitoxantrone followed by daily pulses of ultrasound (green \blacksquare); locally deployed mitoxantrone-laden alginate gel (red \blacktriangle); daily pulse of ultrasound on a blank, locally deployed alginate gel (black \blacktriangledown); daily local mitoxantrone injections (black \circ); or a locally deployed, mitoxantrone-laden alginate gel subjected to daily pulses of ultrasound (blue \blacklozenge). Ultrasound was applied for 2.5 min. (D) Growth curves for human MDA-MB-231 breast cancer xenografts in Nu/J mice. Tumors were left untreated (black curve), exposed to a standard alginate gel loaded with mitoxantrone but not ultrasound (red curve), or treated with a combination of mitoxantrone-laden hydrogels and daily ultrasound (blue curve). Tumor size differences were statistically significant in the mitoxantrone-laden gels treated with daily ultrasound vs. the untreated control from day 7 until the end of the study, and vs. the mitoxantrone-laden hydrogel from day 7 until the end of the study with the exception of days 24–31 and days 59–63. (E) Survival curves for mice left untreated between 70 and 180 d following tumor implantation, after receiving no treatment (black curve), treatment with a mitoxantrone-laden gel but no ultrasound (red curve), or treatment with a combination of mitoxantrone-laden gel and daily ultrasound treatment (blue curve). Kaplan–Meier analysis revealed a statistically significant survival improvement in mice treated with either drug-laden gels or with drug-laden gels exposed to a daily pulse of ultrasound, compared with mice receiving no therapy. (F) Images of tumors explanted at the end of the study from nonsurviving mice (no treatment) and surviving mice (drug alone and drug-laden gel treated with daily ultrasound pulse). Error bars: SEM, $n = 5$ –8 (short-term tumor growth studies and TUNEL staining; $**P < 0.05$ compared with tail vein injection, Holm–Bonferroni test) or $n = 10$ (long-term tumor growth studies and survival studies). Scale bar (F): 1 cm.

xenograft tumors to be implanted directly into the mammary gland (i.e., orthotopic models) or would enable the use of mouse models in which breast tumors develop spontaneously (26, 27). In the present study, the mammary gland was avoided because the distance between the skin and lungs in the mouse is very short compared with the distance between these tissues in humans.

Alginate gels previously were used clinically and have been tested by numerous investigators in animal models, supporting the eventual application of this material in clinical use. As demonstrated here, other types of hydrogels (or appropriate carrier molecules) may be necessary for pulsatile delivery of molecules with substantially different charge or structure compared with mitoxantrone (e.g., naproxen). Applications that may benefit from intermittent high pulsing (e.g., cancer therapy, pain management) or from applications that require release of a drug or factor at specific time points (e.g., in tissue engineering) might benefit greatly from the described system.

Together, the results presented here indicate that self-healing, ionically cross-linked polymers subject to ultrasonic irradiation can trigger on-demand drug delivery in vivo and augment the effects of sustained, baseline release. We also demonstrate a potential therapeutic application of this technology. Combining mitoxantrone with other drugs or macromolecules [e.g., siRNAs (28)] likely will further enhance the therapeutic efficacy of this system. We expect this technology to be applied to many polymers that form ionically or physically cross-linked gels, enabling externally triggered release of a wide variety of factors, including small molecules, proteins, nucleic acids, and nanoparticles (29–31). Beyond clinical applications, this technique also may be useful in fundamental studies of how the delivery profile or timing of application of bioactive agents modulates tissue development, pathology, and regeneration.

Materials and Methods

Alginate Hydrogels and Analysis of Self-Healing. Medical grade, high guluronic acid-content, high M_r alginate (MVG) was purchased from FMC Biopolymers. Alginates were dialyzed against water, filtered (0.22- μ m mesh), freeze dried, and resuspended into Dulbecco's PBS (dPBS; Invitrogen). For drug, protein, and pDNA release studies, the alginate polymers first were combined with the molecule of interest to facilitate noncovalent interactions. Next, alginate was cross-linked with calcium sulfate (CaSO_4 ; Sigma) to a final concentration of 50 mM Ca^{2+} and 20 mg/mL MVG within gels, and a biopsy punch was used to obtain gels of a defined size and shape. Hydrogels were equilibrated overnight in serum free DMEM without phenol red (Invitrogen). Next, gels were transferred to 15-mL tubes filled with dPBS with an additional 0–13.3 mM Ca^{2+} . Gels were subjected to ultrasound treatment (9.6 mW/cm², 5-min pulses, once per hour). The power and time of ultrasound application were chosen based upon measurements of the temperature of media subjected to ultrasound, and within the range used here, temperature change did not exceed controls (controls were kept at 37 $^{\circ}\text{C}$; ultrasound was done at room temperature, and the media temperature remained <32 $^{\circ}\text{C}$), allowing any change in molecular diffusion coefficients or hydrogel degradation via heat to be ruled out of our analysis. To assess gross structural changes, gels that had been subjected to four ultrasound treatments were photographed and immediately flash frozen in liquid nitrogen. After freeze drying, ultrastructure was assessed using a Zeiss EVO scanning electron microscope at the Harvard Center for Nanoscale Systems. To detect cross-linking changes, gels were subjected to a set number of ultrasound treatments, and elastic modulus was measured using an Instron 3342 mechanical testing apparatus. Immediately after modulus testing, gels were flash frozen and lyophilized to obtain their dry masses.

In Vitro Hydrogel Self-Healing and Release Studies. Drug-, protein-, or pDNA-laden alginate gels were cast. The final concentration of each bioactive agent within gels was as follows: mitoxantrone, 850 μ g/mL of gel; SDF-1 α (Pepro-tech), 20 μ g/mL of gel; pDNA (gWiz GFP plasmid DNA, Aldevron), 100 μ g/mL of gel. Ultrasound was applied at 9.6 mW/cm² for 5 min at intervals of 1 h (mitoxantrone), 2 h (SDF-1 α), or 24 h (pDNA). Release was measured colorimetrically (mitoxantrone; 650 nm), by ELISA (SDF-1 α), or by PicoGreen assay (pDNA).

In Vivo Hydrogel Release Study. For in vivo studies, an injectable formulation of alginate was used, as described in the text. Briefly, low M_r MVG was prepared by irradiating medical grade MVG at 5 Mrad with a cobalt source (23). A binary formulation of 20 mg/mL low M_r MVG with 0.5 mg/mL unmodified MVG was mixed with mitoxantrone (850 μ g/mL of gel), then cross-linked with 4% wt/vol CaSO_4 (1.22 M). In vitro studies were performed to ensure that these gels exhibited on-demand release in response to ultrasound

